Sonia Dhanjal (2010). ***Studies on Bioremediation by metal tolerant bacteria with special emphasis on selenium.*** Ph.D. Thesis. CSIR-IMTECH, Chandigarh/ Jawaharlal Nehru University, New Delhi: India.

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SUMMARY OF THE THESIS

Selenium (Se), a naturally occurring element is essential for various biological systems at low concentrations, as a part of *selenocysteine*, an amino acid involved in active site of various enzymes. This element is toxic to all forms of life at higher concentrations. Therefore, it was interesting to study how simple living single-celled microorganism like bacteria survives and responds to elevated levels of selenite oxyions in their microenvironment.

With this objective, three bacterial strains (i.e., *Ochrobactrum* sp. S-5, *Bacillus licheniformis* JS-2 and *Bacillus cereus* CM100B) isolated from the soil rich in selenium content were studied for their selenite reduction ability. Selenium biotransformation potential of each isolate was determined and *Bacillus cereus* (CM100B) was observed to be highly efficient reducer of selenite oxyions among the three strains. The influence of various abiotic factors like temperature, pH, presence of different oxyions and effect of rhamnolipid biosurfactant on selenite reduction by these isolates was also studied. Maximum reduction of selenite oxyions were achieved at 37°C and pH 7-9. Oxyions like chromate severely inhibited selenite reduction by all the three isolates. However, nitrate/nitrite and sulphate/sulphite oxyions had no significant effect on selenite reduction by the two *Bacillus* species (strain JS-2 and CM100B). As mentioned earlier this suggests that selenium, nitrate and sulphur have different reductive pathways in these bacteria. This may be of prospective advantage if the organism is to be used in bioremediation of Se-contaminated wastewater, which often contains high levels of nitrate ions. Many selenate/ selenite respiring bacteria reduce these ions via nitrate reductases. Therefore, nitrate ions must be first removed so that dissimilatory reduction of selenium oxyions can occur from selenium laden wastes (water or sediment). The nitrate ion is thermodynamically more preferred terminal electron acceptor and so the selenium oxyions do not undergo reduction until all the nitrate ions are utilized by the bacteria. The effluents are usually treated

under aerobic conditions as anaerobic conditions are difficult to maintain, the ability of *Bacillus cereus* (strain CM100B) to grow in the presence of oxygen and rapidly reduce Se oxyanions could be extremely advantageous in this respect. Biosurfactant rhamnoilipid was also added to the growth medium to determine its effect on selenite reduction. However, in our study, no significant difference could be observed in selenite reduction in the presence of rhamnolipid biosurfactant. A direct relation between high metal tolerance and antibiotic resistance could only be derived in *Ochrobactrum* sp. (strain S-5) out of the three isolate studied for metal tolerance and antibiotic susceptibility. Of the 21 antibiotics tested, *Ochrobactrum* sp. (strain S-5) was resistant to 15 antibiotics i.e; approximately 71% of the tested antibiotics.

As stated earlier, in the process of biotransformation not only the metal gets transformed but the microbe involved in the process is also affected. To study the effect of toxic selenite oxyions on the microbes involved in the process of selenite reduction some studies were performed to assess the various morphological and physiological changes. In the present study, it was observed that the two *Bacillus* species (strain JS-2 and CM100B) secreted exopolysaccharides when exposed to toxic selenite ions which probably suggests similar mechanism to counteract the toxicity of selenite ions. This relationship of selenite ions and exopolysacchaide production by the bacterial isolates demands further understanding of the concept before any conclusions can be drawn. Also, there are only few reports which show the effect of selenite ions on the process of cell division and subsequently the cell size. Therefore, an attempt was made to study the effect of toxic selenite ions on the cell size of the bacterial isolates. Marked effect on bacterial cell size was observed only in case of *Bacillus cereus* (strain CM100B). This may probably be the result of spore formation in the selenite stressed cells which is also corroborated with the microarray data which shows the up regulation

of various sporulation genes in the selenite stressed cells. However, *Ochrobactrum* sp. (strain S-5) and *Bacillus licheniformis* (strain JS-2) were not affected by toxic selenite ions at the studied selenite concentration. The cell wall fatty acid composition of the bacterial isolates grown in the presence of selenite oxyions indicated a noticeable difference as compared to the control. An increase in ratio of saturated fatty acids to unsaturated fatty acids was observed in all the three bacterial isolates probably suggesting the increase in membrane fluidity. Generally, the cellular fatty acid composition is a result of a sum of complex phenomena maintaining optimal viability of the cell under various conditions. Therefore, it is difficult to understand the adjustment mechanisms linking fatty acid composition to metal stress or any other stress factors. All these morphological and physiological changes observed in the selenite stressed cells show the various strategies adapted by the microbes to cope with the toxicity of the selenite ions.

Scanning electron microscopic studies were carried out to study the morphology of the selenite stressed cells. Interestingly, in *Bacillus cereus* (strain CM100B) several spherical nanospheres of size ~200 nm were found as free deposits and also present as aggregates attached to bacterial cell mass. Similar deposits were also observed in *Bacillus licheniformis* (strain JS-2). The Energy Dispersive X-ray (EDX) analysis of the spherical particles produced specific selenium absorption peaks at 1.37 keV (peak SeLα), 11.22 keV (peak SeKα) and 12.49 keV (peak SeKβ). The ultrastructural intracellular studies of the bacterial isolates revealed the cytoplasmic as well as membrane accumulation of reduced elemental selenium (Se^0). This also led to the increased granularity of the cells as determined by side scatter in flow cytometric analysis.

Bacillus cereus (strain CM100B), was of special interest as it rapidly reduced the toxic selenite oxyions to red elemental selenium nanospheres. Several potential advantages revolve around the

microbes ability to grow in aerobic conditions which include rapid generation of more number of bacterial cells within a short time period under less stringent culture conditions. The aerobically produced nanoparticles by the microbe *Bacillus cereus* (strain CM100B) have been characterized. Biosynthesis of amorphous Se^0 nanospheres under aerobic conditions offers advantages over chemical processes. The strain tolerates high levels of selenium oxyions and generates extracellular nanospheres of selenium (~200 nm in diameter) which can be easily separated from the bacterial biomass by a simple centrifugation step without any post preparative treatment. The amorphous selenium nanospheres formed during the aerobic detoxification of selenite by the strain CM100B were observed to be highly stable due to the presence of high negative charge of -46.86 mV. This green route of biosynthesis of selenium nanospheres is a simple, economically viable and an eco-friendly process resulting in nearly mono-dispersed highly stable selenium nanospheres. Further studies would determine if the diverse properties of the biologically based selenium nanospheres are comparable to chemically synthesized selenium nanoparticles and whether biologically synthesized selenium nanoparticles have practical applications in the field of nanotechnology, biotechnology and environmental sciences.

The strain CM100B was observed to be tolerant against various toxic metals and metalloids which are common environmental pollutants. This multi- metal tolerance indicates that this isolate would have better adaptability and survival rate in the natural environments if used for bioremediation of metal contaminated sites. However, further field studies considering the influence of indigenous microflora of the site on this isolate may probably prove its bioremediation potential.

To further gain insights into the metal-microbe interaction mechanism, FT-IR studies were performed. The FT-IR spectra revealed the presence of numerous functional groups on bacterial cell surface

which are involved in metal binding by the bacterial isolate, *Bacillus cereus* (strain CM100). The FT-IR characteristic peaks corresponded to carboxyl, amide and phosphate groups of bacterial cell surface and cell associated polysaccharides as the dominant functional groups involved in microbe-metal interaction in this bacterium.

In a preliminary study on bacterial swarming, metal and metalloid ions were observed to inhibit the motility of the microbe. The effect on bacterial flagella by the toxic selenite and arsenite ions was revealed by the up regulation of few genes involved in flagellar assembly in the transcriptome analysis of this bacterium. However, to further elucidate the relationship between metals and motility of the microbe, the growth and motility of the bacterium must be simultaneously studied under the influence of metal ions alone, EDTA-metal and surfactin-metal complex.

The global gene expression analysis of bacterial stress response to elevated concentrations of selenium and arsenic oxyions was carried out to investigate the mechanism underlying bacterial tolerance to these toxic metalloids. Data analysis revealed that various genes related to metabolic pathways, transcription, DNA repair and replication and oxidative stress were activated symptomatic of the various stress related responses to toxic selenite ions. It was observed that in order to cope with the oxidative stress that results from the exposure to oxidants like selenite and arsenite oxyions, bacteria have evolved multiple and often redundant defense systems to eliminate the reactive oxyion species (ROS) from the cell. These include thiol system and superoxide dismutases which were found to be highly up regulated in selenite stressed cells. The sporulation process seemed to be triggered in the presence of selenite and arsenite oxyions. Spore formation is another defense mechanism adopted by these microbes to escape the stressful environmental conditions.

The arsenite oxyions proved to be more toxic to the strain at two fold lower concentration as indicated by the overall low cellular

activity of arsenite stressed cells. This bacterium probably survives the toxic arsenite ions by expelling out the arsenite ions with the aid of ACR3, an efflux pump which is an integral membrane protein. However, no specific selenite transport protein was identified in this bacterium. Therefore, it can be speculated that more than one pathway is involved in reduction of selenite in this bacterial isolate.

As there was no conclusive evidence of any specific pathway involved in reduction of selenite oxyions in *Bacillus cereus* in our study, an attempt was made to localize the selenite reduction activity in this bacterium. It was observed that selenite reduction activity was mainly membrane associated. It can be thus concluded that there is involvement of some membrane proteins (may be reductases) which transform the toxic selenite ions to nontoxic elemental state. This may function as the detoxification mechanism and the toxicity of selenite ions is nullified in the cell membrane before these ions actually enter the cytoplasmic region. The assimilatory mechanism is involved in reduction of toxic selenite ions as revealed by the hyperactive protein synthesis machinery of the cell in the presence of selenite oxyions. The activation of various transcriptional networks of metabolic pathway, oxidative stress response, cell division and sporulation cooperate to protect the cell from the toxic selenium oxyions. It can be concluded that multiple mechanism are involved in detoxification of selenite oxyions in this common soil bacterium, *Bacillus cereus* (strain CM100B).

The relative ubiquity of pathways and processes involving reduction of selenium oxyions in archaeal and bacterial strains indicate that they have been important selective factors in microbial evolution. The increasing availability of genome data will help researchers to explore metabolic diversity in these organisms, possibly leading to the discovery of new pathways and regulatory elements. Better tools for *in silico* identification of various selenoproteins are needed. Additional key areas that need further investigation are the

processes and enzymes involved in methylation and demethylation of selenium and the identification of selenite/ selenide reductases in selenium transforming microorganisms.

Lastly, in the present study, two novel species of bacteria, *Yaniella fodinae* ($G5^T$) and *Agrococcus carbonis* ($G4^T$) belonging to family *Yaniellaceae* and *Microbacteriaceae* respectively, isolated from the coalmine soil were also characterized by polyphasic approach. These bacterial isolates transformed selenium oxyions to red elemental Se^0 and tellurium oxyions to black elemental Te^0, respectively. These two novel isolates were observed to be more tolerant to the metalloids as compared to the metals tested. Further studies are needed to explore the biotransformation capabilities of these two novel microbes.